Structure-Activity Assessment and In-Depth Analysis of Biased Agonism in a Set of Phenylalkylamine 5-HT_{2A} Receptor Agonists – Supporting Information

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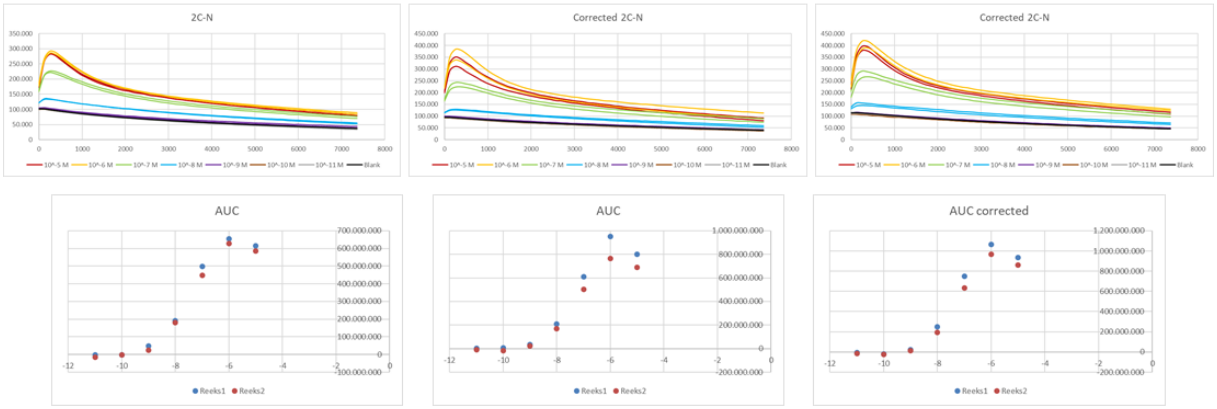
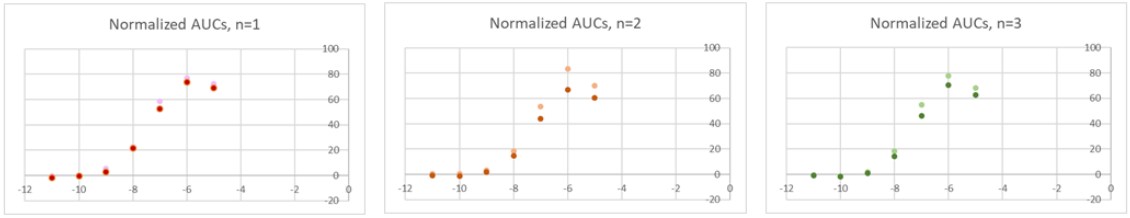
1. SCHEMATIC OVERVIEW OF DATA ANALYSIS

Step 1: calculation of AUC of profiles corrected for inter-well variability and corrected for the blank signal



Step 2: normalization of AUC (with E_{max} of the reference agonist as 100%). Change of reference agonist yields the same EC_{50} value, but different normalized values
Calculated in each individual experiment
Calculated for each respective reference agonist and individual assay



Step 3: pooling of the data: the average values for each concentration point are calculated (mean of the average of each individual experiment)
From these average values, the concentration-response curve is fit

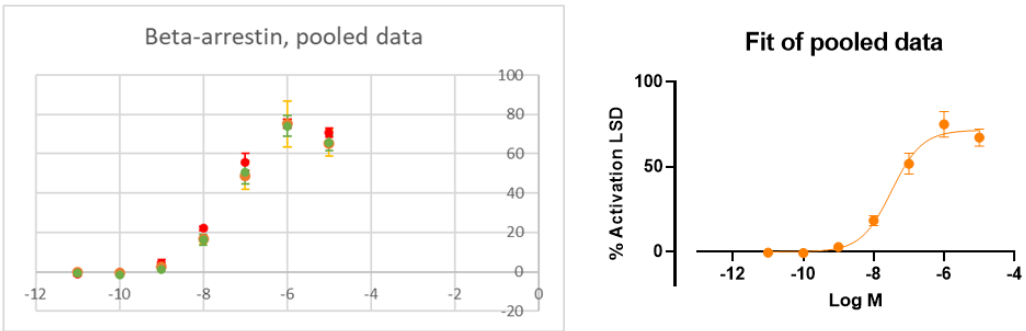


Figure S1: Schematic overview of the steps followed during data analysis in order to obtain EC_{50} and E_{max} values, as reported in the data tables.

2. ASSESSMENT OF RELATIVE 5-HT_{2A}-LGBIT EXPRESSION IN (NON-) SERUM STARVED CELLS

Description of experimental conditions

To assess the influence of the presence of serum on the relative surface expression levels of 5-HT_{2A} in both assays, a flow cytometry experiment was performed. In this experiment, the presence of the HA-tag (N-terminally fused to the 5-HT_{2A}-LgBiT construct) was assessed. Similarly as in the functional assays, HEK 293T cells were seeded in 6-well plates at a density of 500 000 cells per well. Following overnight incubation, the cells were transfected with 3.3 µg of DNA, consisting of either 5-HT_{2A}-LgBiT and pcDNA3.1; 5-HT_{2A}-LgBiT and SmBiT-βarr2; or 5-HT_{2A}-LgBiT and SmBiT-mini$G\alpha_q$. The transfection protocol was identical to that of the functional assays. Several hours post transfection, the medium in which the transfection took place was removed, and the cells were subsequently cultured overnight in either serum-containing DMEM, or in DMEM which does not contain serum. The following day, the cells were detached and washed with flow cytometry buffer (which consists of PBS, 2 % BSA (bovine serum albumin), 2 mM EDTA, and 2 mM NaN_3), and were incubated with 1 µL of mouse anti-HA primary antibody for 30 min at 4 °C. The cells were washed twice with flow cytometry buffer, and incubated with secondary goat anti-mouse antibody, labeled with IR Dye 680 (Li-COR Biosciences). Following additional washing steps, the emitted fluorescence was measured using the CytoFLEX flow cytometer (Beckman Coulter Life Sciences, Brea, USA). Data were processed using FlowJo software version 10 (Ashland, OR, USA).

The data are presented in two panels, of which panel A depicts the control experiments, which assess the background fluorescence captured by the instrument and the aspecific effects introduced by adding antibodies to untransfected cells. As can be seen in the overlaid graphs and the values for median fluorescence intensity (MFI), there is a clear difference between untransfected cells (in black, purple, and gray for no antibodies, only secondary antibody, and primary and secondary antibody present, respectively) and transfected cells (expressing 5-HT_{2A}-LgBiT, depicted by the red curve). This indicates that this protocol is suitable for assessing 5-HT_{2A} cell surface expression. Panel B then comparatively depicts the fluorescence intensity caused by labeling the surface-present HA-tag. Both

from the overlaid curves, where the grey curve is that of the untransfected cells to which both primary and secondary antibody were added, and from the MFI values, it is clear that the fluorescence intensity is comparable for all conditions. More specifically, we compared the MFIs between cells solely expressing 5-HT_{2A}, or expressing 5-HT_{2A} with either βarr2 or miniGα_q, cultured in the presence of serum before performing flow cytometry (similar to the conditions when the standard assay is run), or serum starved overnight. The average of the MFIs, obtained in three independent experiments, revealed that the averaged MFI of serum-starved cells was within 12% of that of non-serum starved cells for all three transfections, with no consistent decrease or increase in any of the conditions. This led us to conclude that we have no reason to believe that possible differences in cell surface expression of the 5-HT_{2A} may have confounded our results.

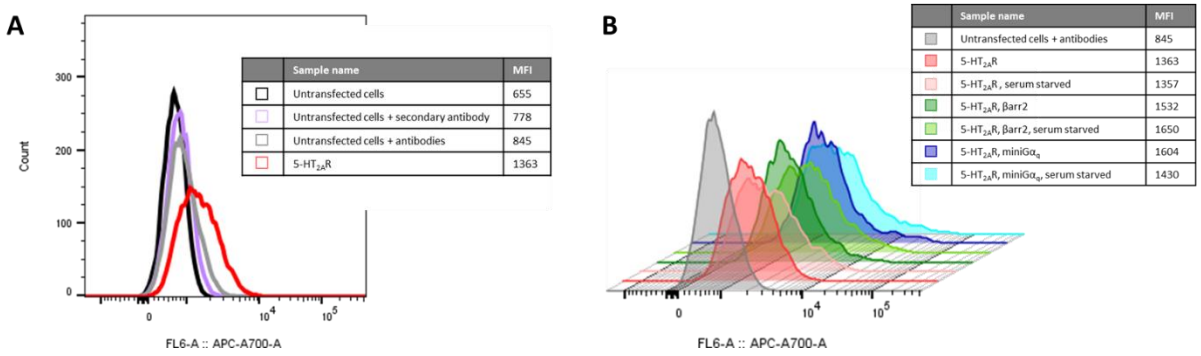


Figure S2: Result of a flow cytometry analysis to assess the surface expression of the HA-tagged 5-HT_{2A}-LgBiT construct, transfected either alone or in combination with SmBiT-βarr2 or SmBiT-miniGα_q, and assessed in HEK 293T cells that were serum starved or not. (**A**) Control experiment, assessing the fluorescence intensity in untransfected cells either without antibody (black), with only secondary antibody (purple), or with both primary and secondary antibodies (gray), and compared to cells transfected with HA-tagged 5-HT_{2A}-LgBiT (red) to which both primary and secondary antibodies were added. (**B**) Comparison of the untransfected cells (with primary and secondary antibody) to the cells transfected with HA-tagged 5-HT_{2A}-LgBiT construct, either alone or in combination with SmBiT-βarr2 or SmBiT-miniGα_q, and either serum starved or not, each condition with both primary and secondary antibody. MFI = median fluorescence intensity. Three independent experiments were performed; the result of a representative experiment is shown.

3. COMPARISON OF ASSAY RESULTS IN CELLS CULTURED IN MEDIUM CONTAINING NO SERUM (SERUM STARVED), DIALYZED SERUM, OR 'NORMAL' FETAL BOVINE SERUM

Description of experimental conditions

The influence of the presence of serum (and thereby serotonin) in routine cell culture conditions on the outcomes of the functional assay monitoring recruitment of βarr2 to the 5-HT_{2A} was assessed. To this end, the previously described cell line stably expressing the components of the 5-HT_{2A}-βarr2 recruitment assay in the NanoBiT® system was used,[1] a system highly similar to the transiently transfected assay format employed to determine structure-activity relationships throughout the manuscript. The cells were routinely cultured in DMEM containing either 'normal' FBS or dialyzed FBS (dFBS, which supposedly does not contain serotonin), and seeded in poly-D-lysine coated 96-well plates 24 hours before the readout. After adherence of the cells, the cells were rinsed with PBS, and were serum starved overnight or not. This yielded three different conditions: (i) cells that were routinely cultured in medium containing 10 % FBS, and were not serum starved overnight (denoted as "non-serum starved cells, FBS"); (ii) cells that were routinely cultured in medium containing 10 % FBS, and were serum starved overnight ("serum starved cells, FBS"); and (iii) cells that were routinely cultured in medium containing 10 % dFBS and were not serum starved overnight ("non-serum starved cells, dFBS"). The following day, the assay was performed as described in the Materials & Methods section, and concentration ranges of LSD, serotonin, and 25D-NBOMe (hence including the three structural classes of serotonergic psychedelics) were included. The resulting concentration-response curves are shown in Figure S3, and the obtained pharmacological parameters in Table S1.

In Figure S3, the upper panels provide a comparison of the normalized (relative to the maximal response of LSD) responses of the different compounds in the different conditions. In the lower panels, the relative comparison is made of the three compounds, each in a different cell culture condition. From the Figure, it can be derived that, for serotonin and 25D-NBOMe, the condition in which the cells were serum starved overnight resulted in consistently higher EC_{50} and (relative) E_{max} values. On the other hand, no significant differences were found between cells cultured in medium containing dialyzed or non-dialyzed serum. These observations were additionally supported by the numerical

values provided in Table S1. Importantly, when comparing the relative activities of the compounds in the different conditions (as is done in the lower panels of Figure S3), it is clear that all conditions yield the same relative ranking in terms of both potency and efficacy. This suggests that, in this specific assay format, with the currently used conditions, no different conclusions would have been drawn when serum starving the cells, or culturing the cells in medium containing dialyzed serum.

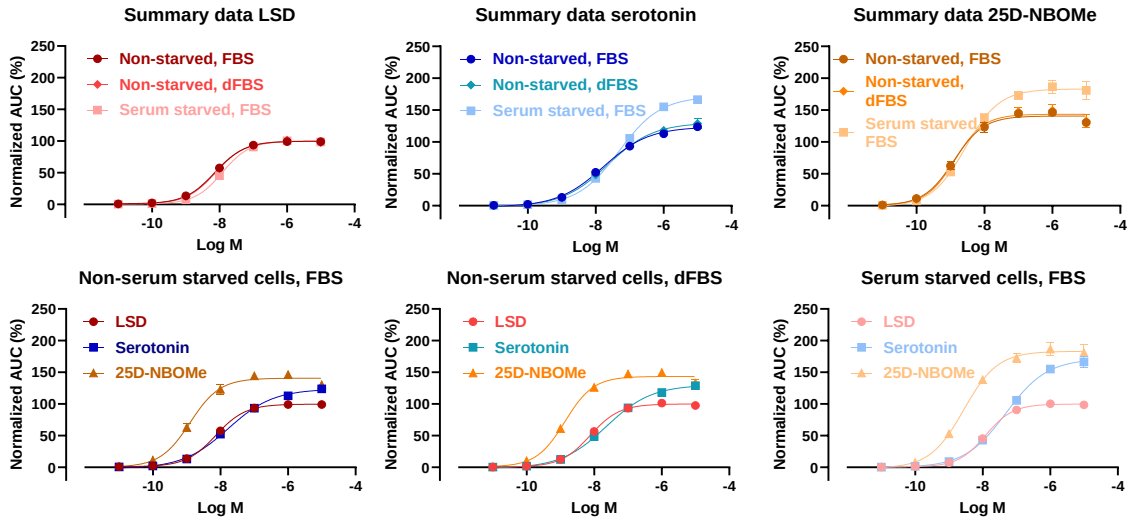


Figure S3: Concentration-response curves of LSD, serotonin, and 25D-NBOMe in the 5-HT_{2A}-βarr assay performed with cells that were cultured in three different culturing conditions, as outlined in the text. Results are from three independent experiments, each performed in duplicate.

Table S1: Potency (EC_{50}) and efficacy (E_{max}, relative to the E_{max} of LSD) measures of LSD, serotonin, and 25D-NBOMe in the 5-HT_{2A}-βarr assay performed with cells that were cultured in three different culturing conditions, as outlined in the text. Results are from three independent experiments, each performed in duplicate.

	EC_{50} (nM)(95% CI)	E_{max} (%)(95% CI)
Cells cultured in medium containing FBS, non-serum starved overnight		
LSD	7.26 (5.55 – 9.37)	99.8 (96.0 – 104)
Serotonin	16.7 (11.7 – 24.3)	124 (117 – 132)
25D-NBOMe	1.26 (0.83 – 1.86)	141 (134 – 148)
Cells cultured in medium containing FBS, serum starved overnight		
LSD	12.0 (10.1 – 14.5)	99.9 (96.8 – 103)
Serotonin	48.7 (34.5 – 69.9)	171 (162 – 183)
25D-NBOMe	2.74 (1.78 – 4.21)	183 (174 – 194)
Cells cultured in medium containing dFBS, non-serum starved overnight		
LSD	7.95 (6.83 – 9.22)	100 (97.8 – 102)
Serotonin	30.8 (25.8 – 37.0)	136 (132 – 140)
25D-NBOMe	1.54 (1.21 – 1.96)	148 (143 – 152)

4. COMPARISON BETWEEN EXPERIMENTAL AND DOCKED BINDING POSE OF 25CN-NBOH

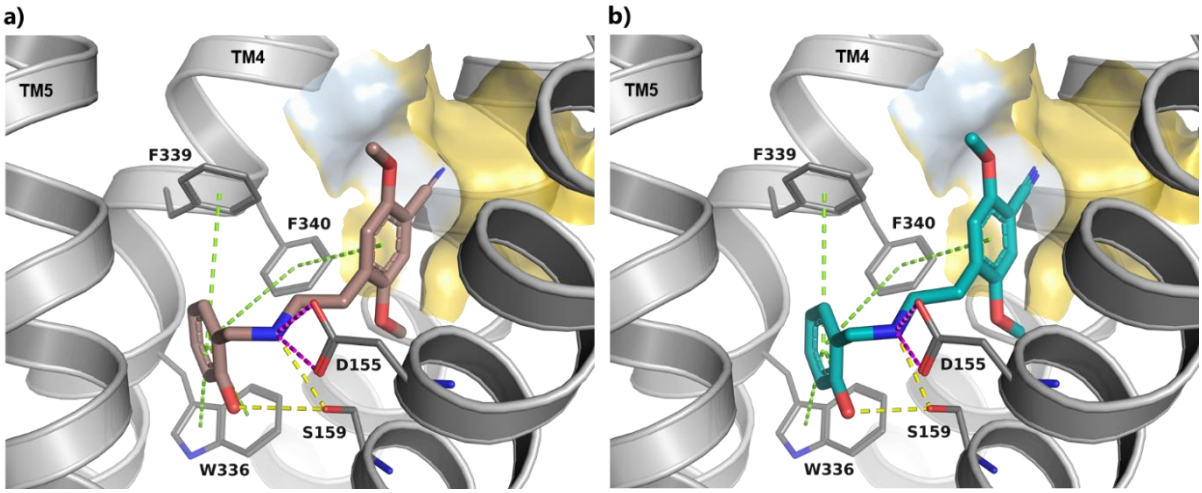
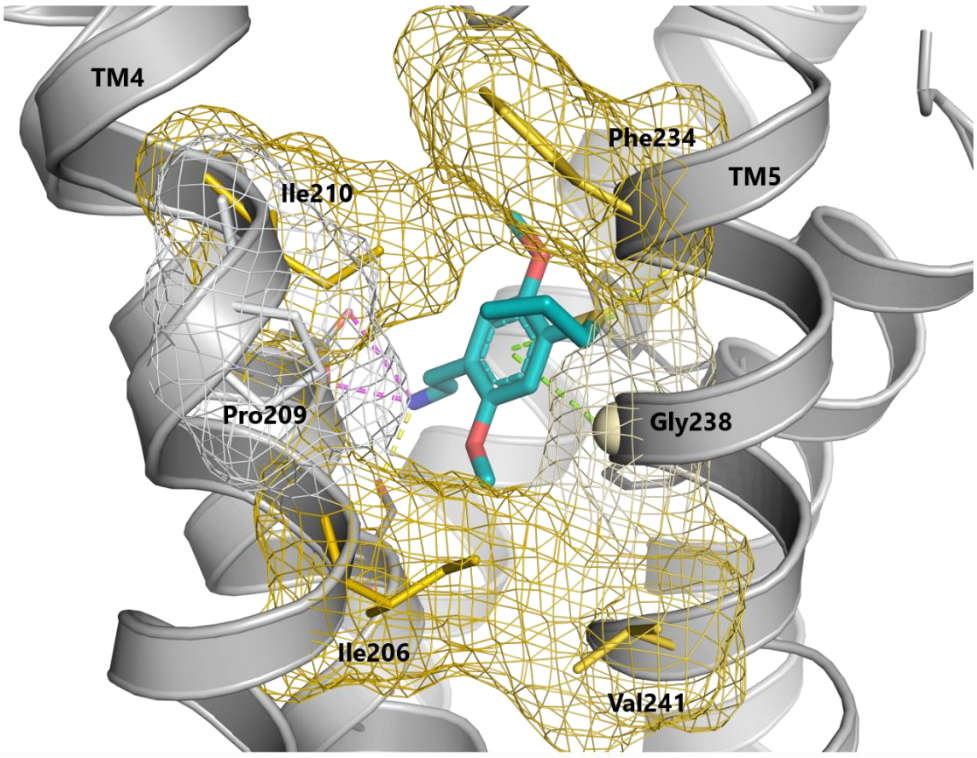


Figure S4. The binding mode of 25CN-NBOH reveals a network of ligand-receptor interactions pivoted by the ligand's *N*-benzyl moiety. Main ligand-receptor interactions and comparison between the experimental (light brown sticks) and redocking binding mode (cyan sticks) of 25CN-NBOH in the optimized cryo-EM structure of the G_q-coupled 5-HT_{2A} (PDB ID 6WHA,[2] gray lines and cartoon). The interactions are displayed as dashed lines and colored in green (aromatic, π-π stacking), yellow (hydrogen bond) and pink (salt-bridge). The receptor hydrophobic sub-pocket between TM4 and TM5 is shown as a surface and colored according to the Eisenberg hydrophobicity scale,[3] from highly hydrophilic (blue) to highly hydrophobic (yellow).

5. ESTIMATED DISTANCES BETWEEN THE DOCKED BINDING POSE OF THE PHENETHYLAMINES AND THE RESIDUES LINING THE HYDROPHOBIC POCKET BETWEEN TM4 AND TM5

a)



b)

Compound	cLogD$_{7.4}$	Ile206	Pro209	Ile210	Phe234	Gly238	Val241
2C-CN	-1.27	9.4	11.6	8.1	7.3	4.6	8.9
2C-N	-1.13	8.7	11.3	8.0	7.9	4.2	8.0
1d	-0.91	7.8	8.5	4.6	6.5	4.9	9.1
2C-D	-0.65	9.0	11.3	7.9	7.7	4.5	8.5
2C-T	-0.52	8.4	10.1	6.6	6.6	4.0	8.5
DOT	-0.28	8.3	10.2	6.8	6.7	3.9	8.3
2C-P	0.24	9.1	10.0	6.0	6.4	5.0	9.6
2C-T-7	0.26	7.8	8.9	4.8	6.7	5.3	9.5
DOT-7	0.49	6.8	7.5	4.0	5.8	4.2	8.4

Figure S5: Residues lining the hydrophobic side pocket between the TM4 and TM5 of the 5-HT$_{2A}$ receptor. B. Shortest distance (in angstrom) between the terminal heavy atom of the R4-substituent in the predicted binding pose of the phenethylamines and the closest side chain heavy atom in the hydrophobic pocket residues.

6. QUANTIFICATION OF THE RESULTS WITH THE 30 MIN AUC (AREA UNDER THE CURVE) VALUES

Results table

Table S2: Data obtained in the βarrestin2 or miniGα_q recruitment assays, expressed with LSD as the reference agonist, and using the first 30 minutes of the time-luminescence profile. The EC_{50} values are a measure of the potency of a substance in the respective assay, the E_{max} values are a measure of the efficacy, where the maximal response of LSD was used for the purpose of normalization. Data are combined from at least three independent experiments, each performed in duplicate.

	β-arrestin 2			**miniGα_q**			
	pEC_{50} (95 % CI)	**EC_{50} (nM)(95 % CI)**	**E_{max} (%)(95 % CI)**	**pEC_{50} (95 % CI)**	**EC_{50} (nM)(95 % CI)**	**E_{max} (%)(95 % CI)**	**β**
LSD	7.35 (7.18 – 7.52)	44.9 (30.3 - 65.7)	99.1 (93.0 – 105)	7.48 (7.26 – 7.69)	33.5 (20.3 – 54.6)	99.3 (92.0 – 107)	0
5-HT	7.87 (7.76 – 7.97)	13.6 (10.6 – 17.5)	122 (118 – 126)	6.99 (6.83 – 7.15)	103 (70.8 – 148)	234 (220 – 248)	0.692
2C-CN (1j)	6.60 (6.45 – 6.75)	250 (177 – 356)	72.3 (68.5 – 76.3)	6.29 (6.04 – 6.55)	514 (280 – 907)	88.2 (79.7 – 97.1)	0.383
2C-N (1g)	7.35 (7.13 – 7.59)	44.6 (26.0 – 73.9)	76.0 (69.9 – 82.2)	7.02 (6.86 – 7.19)	94.7 (63.9 – 139)	105 (98.2 – 112)	0.315
1d	7.38 (7.09 – 7.69)	41.7 (20.5 – 80.5)	82.7 (74.6 – 91.0)	7.05 (6.78 – 7.34)	88.4 (46.0 – 165)	100 (89.5 – 111)	0.359
2C-D (1b)	7.39 (7.05 – 7.76)	41.1 (17.4 – 89.1)	82.7 (74.3 – 91.5)	7.20 (6.92 – 7.50)	63.0 (31.3 – 120)	124 (114 – 135)	0.408
2C-T (1e)	7.76 (7.58 – 7.93)	17.4 (11.8 – 26.2)	94.5 (89.4 – 99.7)	7.44 (7.22 – 7.68)	36.0 (20.9 – 61.0)	127 (118 – 137)	0.314
2C-P (1c)	7.89 (7.62 – 8.16)	12.8 (6.99 – 23.9)	111 (101 – 121)	7.32 (7.06 – 7.59)	48.3 (25.8 – 86.6)	200 (182 – 218)	0.425
2C-T-7 (1f)	8.17 (7.96 – 8.39)	6.79 (4.11 – 10.9)	114 (107 – 122)	7.81 (7.53 – 8.08)	15.6 (8.41 – 29.5)	196 (179 – 214)	0.288
DOT (1h)	7.72 (7.51 – 7.92)	19.2 (12.1 – 31.1)	124 (116 – 132)	7.14 (6.83 – 7.49)	71.9 (32.7 – 148)	215 (194 – 238)	0.457
DOT-7 (1i)	8.47 (8.21 – 8.74)	3.36 (1.82 – 6.13)	124 (115 – 133)	8.01 (7.70 – 8.32)	9.86 (4.76 – 20.1)	229 (207 – 252)	0.326
25N-NBOH (2e)	8.81 (8.57 – 9.05)	1.54 (0.900 – 2.67)	141 (130 – 152)	8.53 (8.21 – 8.84)	2.97 (1.43 – 6.15)	143 (128 – 159)	0.404
25T-NBOH (2a)	8.43 (8.23 – 8.65)	3.69 (2.26 – 5.96)	158 (148 – 168)	8.12 (7.89 – 8.37)	7.55 (4.29 – 12.9)	148 (137 – 160)	0.468
25N-NBMD (2h)	8.42 (8.28 – 8.57)	3.77 (2.69 – 5.26)	140 (133 – 146)	7.88 (7.69 – 8.07)	13.2 (8.57 – 20.3)	116 (108 – 124)	0.783
25N-NBOMe (2f)	8.61 (8.46 – 8.76)	2.45 (1.75 – 3.44)	170 (162 – 179)	8.32 (8.05 – 8.62)	4.74 (2.39 – 9.00)	192 (173 – 213)	0.363
25N-NBF (2g)	7.64 (7.51 – 7.78)	22.8 (16.7 – 31.1)	135 (129 – 141)	7.01 (6.88 – 7.14)	97.7 (71.8 – 133)	106 (99.7 – 113)	0.858
25T-NBOMe (2b)	8.43 (8.22 – 8.65)	3.70 (2.24 – 6.04)	179 (167 – 191)	8.14 (7.87 – 8.42)	7.31 (3.79 – 13.6)	205 (186 – 225)	0.365
25T7-NBOH (2c)	7.53 (7.37 – 7.69)	29.6 (20.6 – 42.6)	167 (156 – 178)	7.50 (7.22 – 7.78)	31.4 (16.5 – 59.8)	159 (141 – 178)	0.232
25T7-NBOMe (2d)	7.70 (7.49 – 7.90)	20.3 (12.5 – 32.6)	174 (161 – 188)	7.54 (7.28 – 7.80)	28.6 (15.7 – 52.4)	176 (159 – 194)	0.274

Overlaid graphs

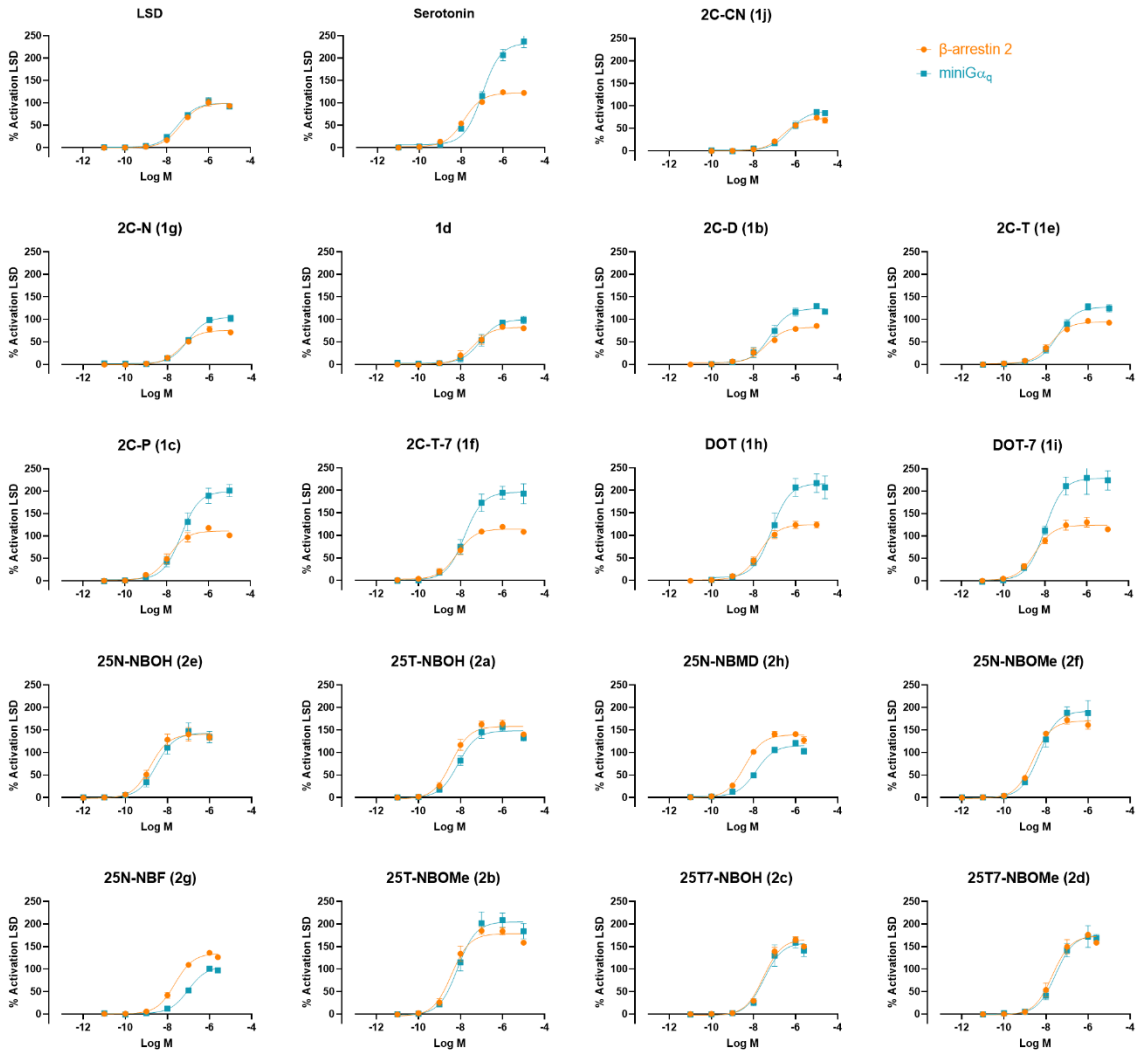


Figure S6: Overlay of the concentration-response curves for each of the test substances in the two assay formats. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean). Curves represent three parametric, non-linear fits, in which LSD is used for normalization, and the first 30 minutes of the time-luminescence profiles are used for the calculation of the AUC.

Calculated bias factors

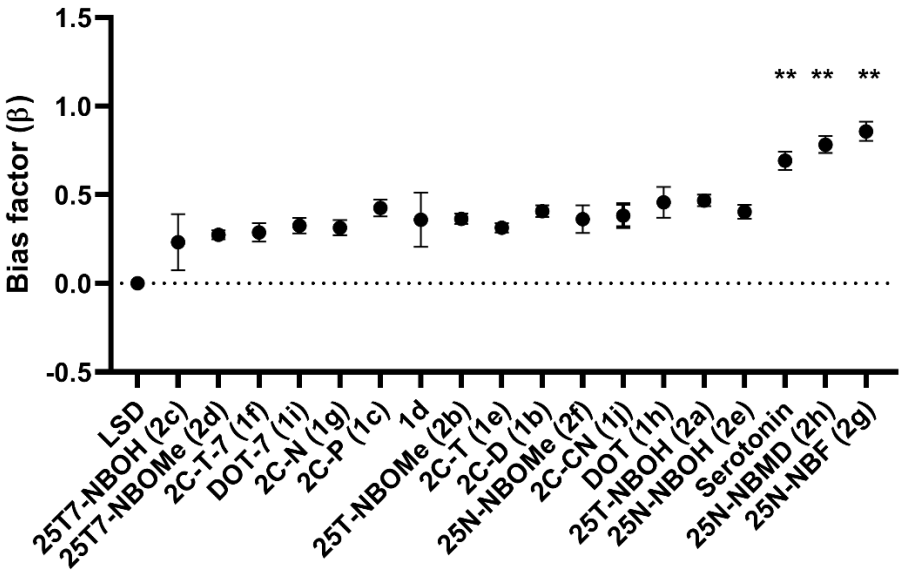


Figure S7: Visual representation of the bias factors (β) ± SEM (standard error of the mean), where ** stands for $p < 0.01$ in the non-parametric Kruskal Wallis analysis of significance. LSD is used as the reference agonist, and the first 30 minutes of the time-luminescence profiles are used for the calculation of the AUC.

Bias plots

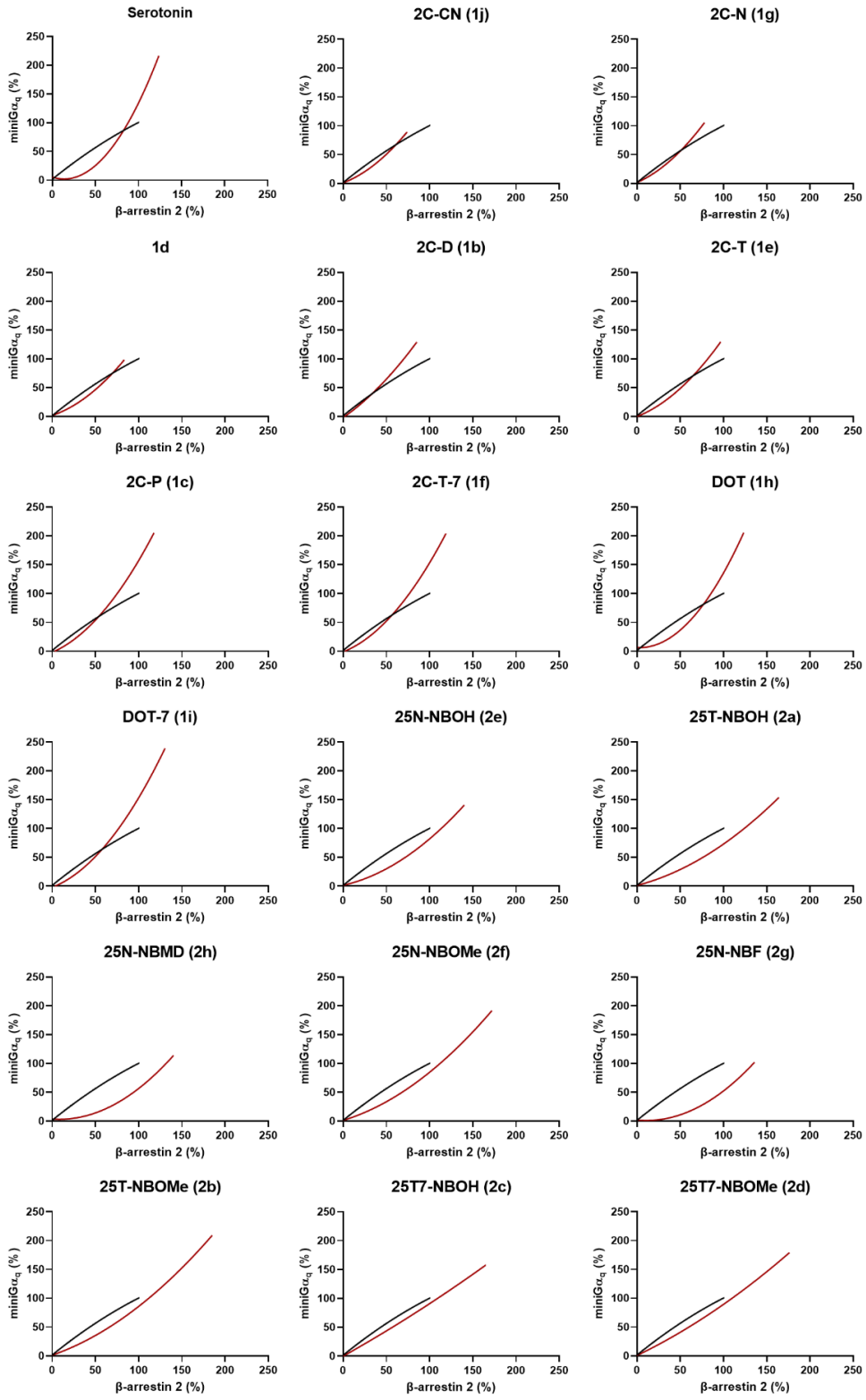


Figure S8: Qualitative bias plots, where each panel shows the centered second order polynomial fit of the activation values at equimolar concentrations of the substance in the respective assays in red, and that of the reference agonist (in this case LSD) in black. The first 30 minutes of the time-luminescence profiles are used for the calculation of the AUC.

7. QUANTIFICATION OF THE RESULTS WITH SEROTONIN AS THE REFERENCE AGONIST

Results Table

Table S3: Data obtained in the βarrestin2 or miniGα_q recruitment assays, expressed with serotonin as the reference agonist, and using the complete 2h time-luminescence profile. The EC_{50} values are a measure of the potency of a substance in the respective assay, the E_{max} values are a measure of the efficacy, where the maximal response of serotonin was used for the purpose of normalization. Data are combined from at least three independent experiments, each performed in duplicate.

	β-arrestin 2			**miniGα_q**			
	pEC_{50} (95 % CI)	**EC_{50} (nM)(95 % CI)**	**E_{max} (%)(95 % CI)**	**pEC_{50} (95 % CI)**	**EC_{50} (nM)(95 % CI)**	**E_{max} (%)(95 % CI)**	**β**
LSD	7.91 (7.69 – 8.14)	12.2 (7.33 - 20.3)	86.0 (79.8 - 92.3)	7.92 (7.66 – 8.18)	12.1 (6.69 - 22.1)	47.2 (43.1 - 51.3)	-0.812
5-HT	8.04 (7.92 – 8.17)	9.10 (6.84 - 12.1)	99.0 (95.4 - 103)	7.06 (6.91 – 7.22)	86.5 (60.0 - 123)	99.5 (93.8 - 105)	0
2C-CN (1j)	6.76 (6.61 – 6.90)	175 (126 - 246)	67.2 (63.9 - 70.6)	6.29 (6.03 – 6.57)	517 (268 - 944)	37.3 (33.7 - 41.2)	-0.233
2C-N (1g)	7.50 (7.21 – 7.78)	32.0 (16.5 - 61.6)	65.7 (59.5 - 72.2)	7.03 (6.86 – 7.21)	92.8 (61.6 - 138)	46.5 (43.3 - 49.8)	-0.371
1d	7.57 (7.27 – 7.87)	26.8 (13.6 - 53.6)	71.2 (64.6 - 78.1)	7.13 (6.80 – 7.48)	74.5 (33.1 - 157)	44.7 (39.4 - 50.3)	-0.356
2C-D (1b)	7.78 (7.52 – 8.01)	16.8 (9.67 - 30.3)	78.7 (72.9 - 84.6)	7.23 (6.92 – 7.57)	59.6 (27.2 - 122)	56.4 (51.2 - 61.8)	-0.306
2C-T (1e)	7.90 (7.59 – 8.19)	12.8 (6.52 - 25.6)	75.2 (68.5 - 82.0)	7.44 (7.19 – 7.69)	36.8 (20.6 - 64.3)	54.8 (50.5 - 59.2)	-0.383
2C-P (1c)	7.99 (7.69 – 8.30)	10.2 (5.07 - 20.3)	97.6 (88.4 - 107)	7.42 (7.16 – 7.70)	37.6 (19.9 - 69.3)	85.3 (77.8 - 93.1)	-0.365
2C-T-7 (1f)	8.31 (8.05 – 8.59)	4.96 (2.60 - 9.00)	94.9 (88.1 - 102)	7.86 (7.56 – 8.14)	13.9 (7.21 - 27.3)	91.3 (82.9 - 100)	-0.476
DOT (1h)	7.86 (7.58 – 8.12)	14.0 (7.56 - 26.4)	90.6 (83.0 - 98.4)	7.18 (6.84 – 7.56)	66.0 (27.8 - 144)	86.7 (77.4 - 96.2)	-0.289
DOT-7 (1i)	8.67 (8.36 – 8.96)	2.12 (1.10 - 4.33)	97.3 (90.1 - 105)	8.13 (7.83 – 8.44)	7.47 (3.62 - 14.7)	98.8 (89.8 - 108)	-0.461
25N-NBOH (2e)	9.18 (8.91 – 9.47)	0.660 (0.342 - 1.23)	122 (112 - 133)	8.76 (8.44 – 9.07)	1.73 (0.85 - 3.61)	59.7 (53.5 - 66.1)	-0.247
25T-NBOH (2a)	8.73 (8.42 – 9.03)	1.85 (0.934 - 3.78)	125 (113 – 136)	8.35 (8.10 – 8.63)	4.43 (2.36 - 8.04)	59.9 (55.3 - 64.6)	-0.278
25N-NBMD (2h)	8.70 (8.53 – 8.86)	2.00 (1.38 - 2.94)	126 (120 - 132)	8.02 (7.81 – 8.25)	9.45 (5.69 - 15.5)	51.4 (47.8 - 55.1)	0.0890
25N-NBOMe (2f)	8.96 (8.81 – 9.11)	1.09 (0.773 - 1.54)	156 (148 - 164)	8.60 (8.38 – 8.81)	2.53 (1.55 - 4.19)	81.4 (75.7 - 87.2)	-0.328
25N-NBF (2g)	7.85 (7.70 – 7.99)	14.3 (10.4 - 19.8)	119 (113 - 124)	7.11 (6.93 – 7.31)	77.3 (49.6 - 118)	46.2 (42.7 - 50.0)	0.156
25T-NBOMe (2b)	8.71 (8.41 – 9.00)	1.96 (1.01 - 3.89)	141 (128 - 154)	8.38 (8.08 – 8.69)	4.17 (2.04 - 8.25)	80.9 (73.3 - 88.8)	-0.409
25T7-NBOH (2c)	7.84 (7.68 – 8.00)	14.4 (10.1 - 20.8)	143 (135 - 152)	7.75 (7.46 – 8.02)	17.9 (9.49 - 34.3)	73.7 (66.1 - 81.7)	-0.558
25T7-NBOMe (2d)	8.10 (7.91 – 8.29)	7.99 (5.08 - 12.4)	148 (138 - 158)	7.92 (7.68 – 8.15)	12.1 (7.12 - 20.8)	77.6 (71.3 - 84.1)	-0.486

Overlaid graphs

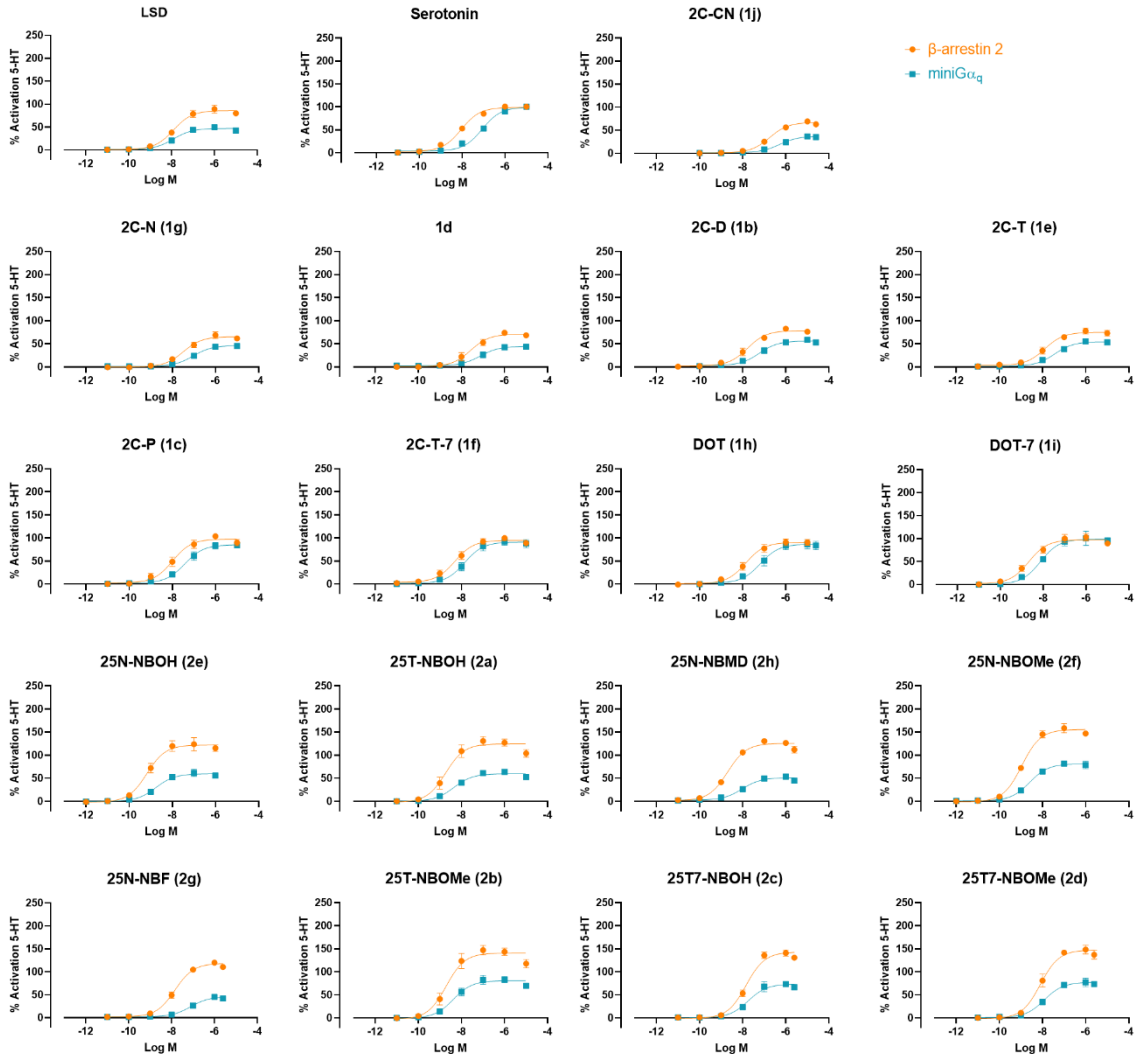


Figure S9: Overlay of the concentration-response curves for each of the test substances in the two assay formats. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean). Curves represent three parametric, non-linear fits, normalized to the maximal response of serotonin, and using the entire (2h) time-luminescence profiles for the calculation of the AUC.

Calculated bias factors

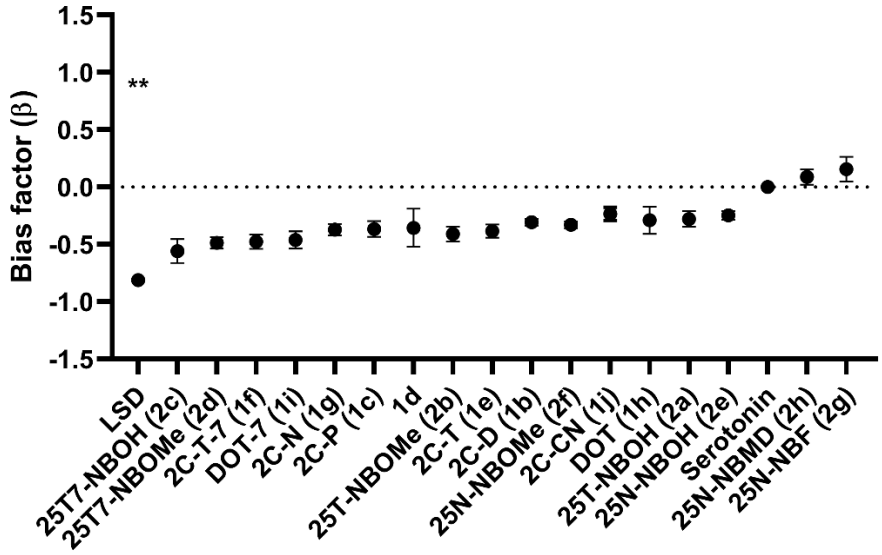


Figure S10: Visual representation of the bias factors (β) ± SEM (standard error of the mean), where ** stands for p< 0.01 in the non-parametric Kruskal Wallis analysis of significance. Serotonin is used as the reference agonist, and the entire (2h) time-luminescence profiles are used for the calculation of the AUC.

Bias plots

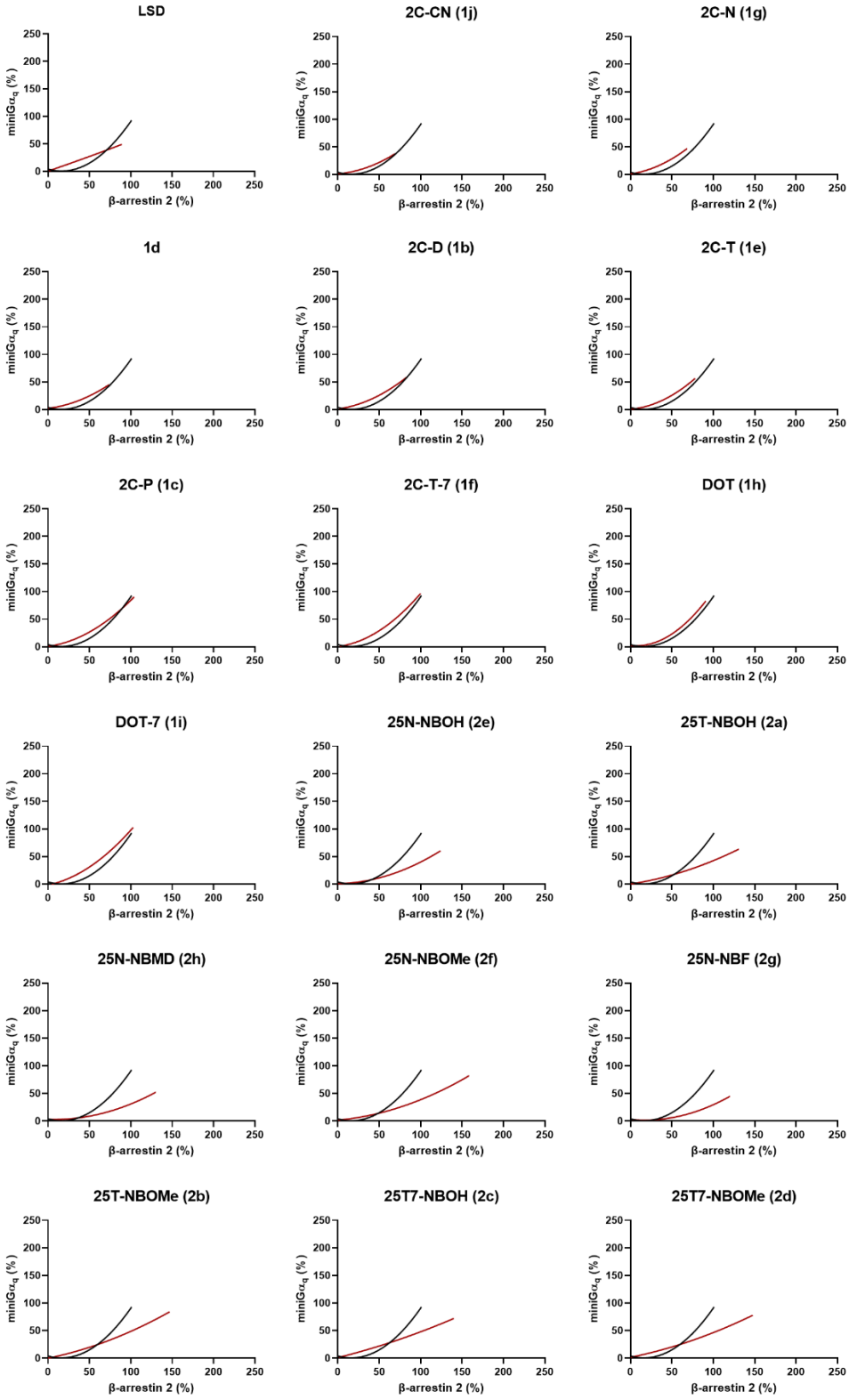


Figure S11: Qualitative bias plots, where each panel shows the centered second order polynomial fit of the activation values at equimolar concentrations of the substance in the respective assays in red, and that of the reference agonist (in this case serotonin) in black. The entire (2h) time-luminescence profile is used for the calculation of the AUC.

8. QUANTIFICATION OF THE RESULTS WITH THE 30 MIN AUC (AREA UNDER THE CURVE) VALUES AND SEROTONIN AS A REFERENCE AGONIST

Results table

Table S4: Data obtained in the βarrestin2 or miniGα_q recruitment assays, expressed with serotonin as the reference agonist, and using the first 30 minutes of the time-luminescence profile. The EC_{50} values are a measure of the potency of a substance in the respective assay, the E_{max} values are a measure of the efficacy, where the maximal response of serotonin was used for the purpose of normalization. Data are combined from at least three independent experiments, each performed in duplicate.

	β-arrestin 2			miniGα_q			
	pEC_{50} (95 % CI)	**EC_{50} (nM)(95 % CI)**	**E_{max} (%)(95 % CI)**	**pEC_{50} (95 % CI)**	**EC_{50} (nM)(95 % CI)**	**E_{max} (%)(95 % CI)**	**β**
LSD	7.35 (7.18 – 7.53)	44.6 (29.3 – 66.9)	81.2 (75.8 – 86.6)	7.48 (7.26 – 7.70)	33.2 (20.0 – 54.7)	42.2 (39.1 – 45.4)	-0.877
5-HT	7.88 (7.78 – 7.99)	13.1 (10.3 – 16.7)	99.2 (95.9 – 103)	6.99 (6.83 – 7.16)	101 (69.7 – 146)	99.2 (93.2 – 105)	0
2C-CN (1j)	6.60 (6.46 – 6.74)	250 (183 – 345)	62.6 (59.6 – 65.7)	6.26 (6.01 – 6.54)	546 (288 – 988)	35.3 (31.7 – 39.1)	-0.290
2C-N(1g)	7.35 (7.13 – 7.59)	44.5 (25.5 – 75.0)	65.6 (60.2 – 71.3)	7.02 (6.86 – 7.18)	96.1 (66.5 – 138)	44.5 (41.7 – 47.4)	-0.390
1d	7.38 (7.10 – 7.68)	42.0 (20.9 – 80.3)	71.4 (64.6 – 78.6)	7.07 (6.75 – 7.40)	85.8 (40.0 – 177)	42.3 (37.2 – 47.7)	-0.346
2C-D (1b)	7.67 (7.41 – 7.91)	21.5 (12.3 – 39.0)	75.0 (69.3 – 80.9)	7.20 (6.87 – 7.56)	63.6 (27.9 – 135)	53.2 (47.9 – 58.6)	-0.296
2C-T (1e)	7.76 (7.53 – 7.98)	17.4 (10.4 – 29.9)	70.0 (65.0 – 75.2)	7.45 (7.23 – 7.67)	35.8 (48.4 – 55.9)	52.1 (48.4- 55.9)	-0.446
2C-P (1c)	7.89 (7.61 – 8.17)	12.8 (6.81 – 24.4)	96.1 (87.3 – 105)	7.31 (7.06 – 7.58)	49.2 (26.4 – 87.7)	84.4 (76.9 – 92.2)	-0.279
2C-T-7 (1f)	8.17 (7.95 – 8.41)	6.73 (3.89 – 11.3)	91.1 (84.9 – 97.4)	7.81 (7.50 – 8.11)	15.4 (7.75 – 31.3)	87.9 (79.3 – 96.9)	-0.478
DOT (1h)	7.73 (7.49 – 7.96)	18.6 (11.0 – 32.3)	91.5 (84.8 – 98.5)	7.15 (6.86 – 7.46)	70.9 (34.5 – 138)	87.7 (79.6 – 95.9)	-0.303
DOT-7 (1i)	8.49 (8.23 – 8.74)	3.25 (1.80 – 5.84)	98.5 (91.9 – 105)	8.01 (7.71 – 8.32)	9.78 (4.78 – 19.7)	102 (92.6 – 112)	-0.106
25N-NBOH (2e)	8.82 (8.57 – 9.06)	1.53 (0.879 – 2.70)	123 (113 – 133)	8.53 (8.25 – 8.80)	2.99 (1.60 – 5.58)	60.4 (54.7 – 66.2)	-0.298
25T-NBOH (2a)	8.44 (8.21 – 8.68)	3.60 (2.10 – 6.13)	117 (109 – 125)	8.12 (7.90 – 8.35)	7.61 (4.50 – 12.6)	60.7 (56.4- 65.1)	-0.290
25N-NBMD (2h)	8.42 (8.29 – 8.56)	3.77 (2.73 – 5.18)	122 (116 – 127)	7.87 (7.67 – 8.06)	13.6 (8.81 – 21.3)	49.7 (46.5 – 53.1)	0.0796
25N-NBOMe (2f)	8.61 (8.46 – 8.76)	2.45 (1.73 – 3.48)	149 (141 – 157)	8.32 (8.10 – 8.56)	4.78 (2.79 – 7.96)	81.0 (74.4 – 87.8)	-0.337
25N-NBF (2g)	7.65 (7.51 – 7.79)	22.6 (16.4 – 31.3)	117 (111 – 122)	6.98 (6.82 – 7.15)	104 (70.9 – 152)	46.1 (42.7 – 49.6)	0.158
25T-NBOMe (2b)	8.42 (8.20 – 8.66)	3.78 (2.21 – 6.39)	134 (124 – 144)	8.14 (7.88 – 8.41)	7.28 (3.87 – 13.3)	83.7 (76.1 – 91.4)	-0.393
25T7-NBOH (2c)	7.50 (7.38 – 7.62)	31.6 (23.8 – 42.0)	136 (129 – 143)	7.50 (7.22 – 7.77)	32.0 (16.8 – 60.7)	71.7 (63.6 – 80.2)	-0.230
25T7-NBOMe (2d)	7.66 (7.50 – 7.80)	22.1 (15.8 – 31.4)	142 (134 – 150)	7.52 (7.29 – 7.76)	30.0 (17.6 – 51.3)	79.7 (72.8 – 86.9)	-0.393

Overlaid graphs

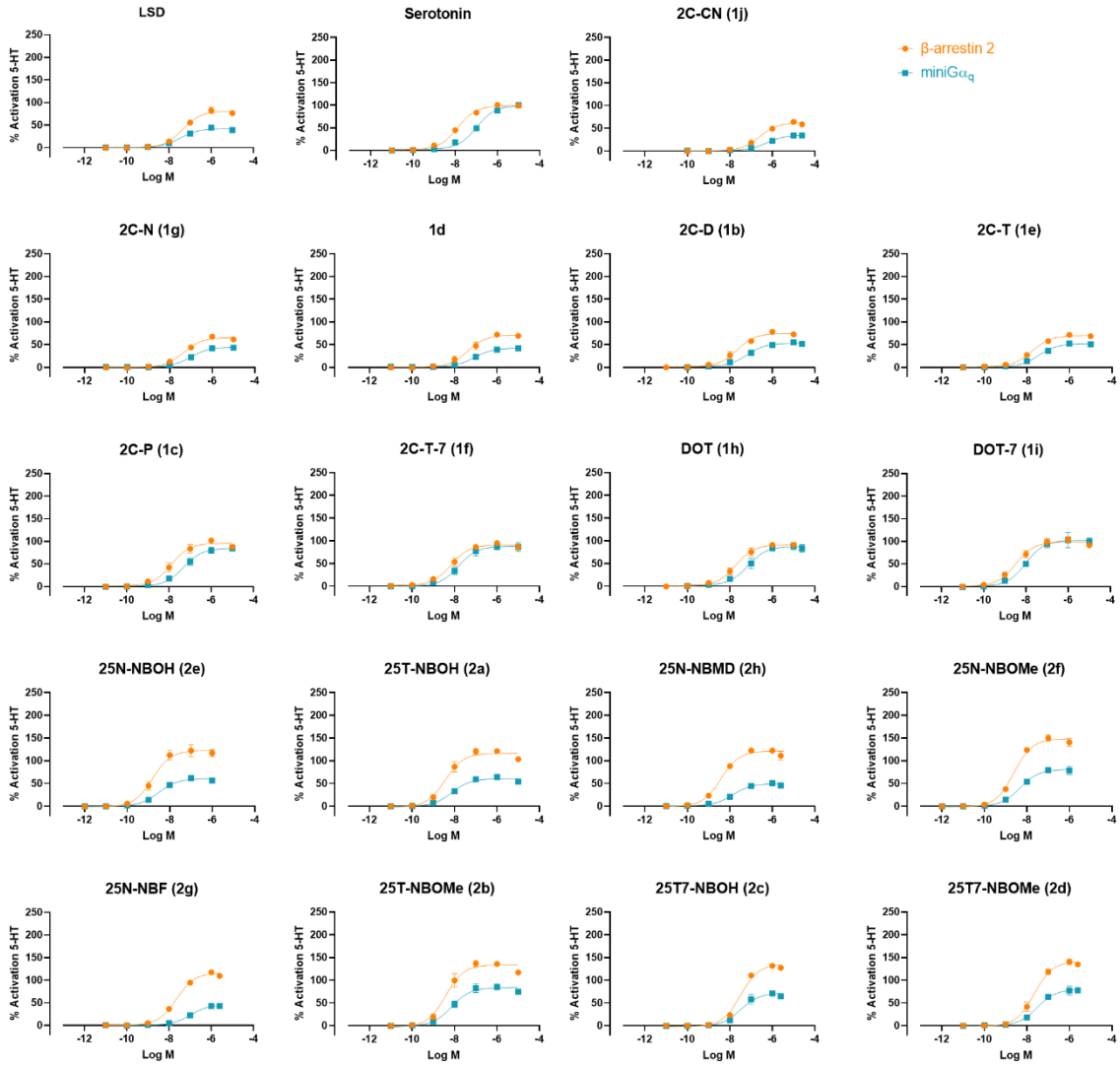


Figure S12: Overlay of the concentration-response curves for each of the test substances in the two assay formats. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean). Curves represent three parametric, non-linear fits, normalized to the maximal response of serotonin, where the first 30 minutes of the time-luminescence profiles are used for the calculation of the AUC.

Calculated bias factors

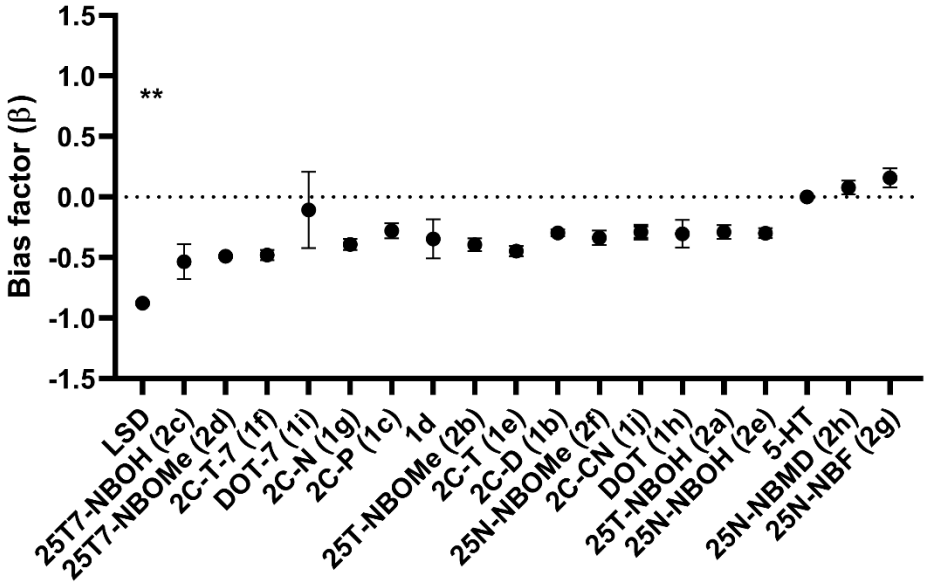


Figure S13: Visual representation of the bias factors (β) ± SEM (standard error of the mean), where ** stands for $p < 0.01$ in the non-parametric Kruskal Wallis analysis of significance. Serotonin is used as the reference agonist, and the first 30 minutes of the time-luminescence profiles are used for the calculation of the AUC.

Bias plots

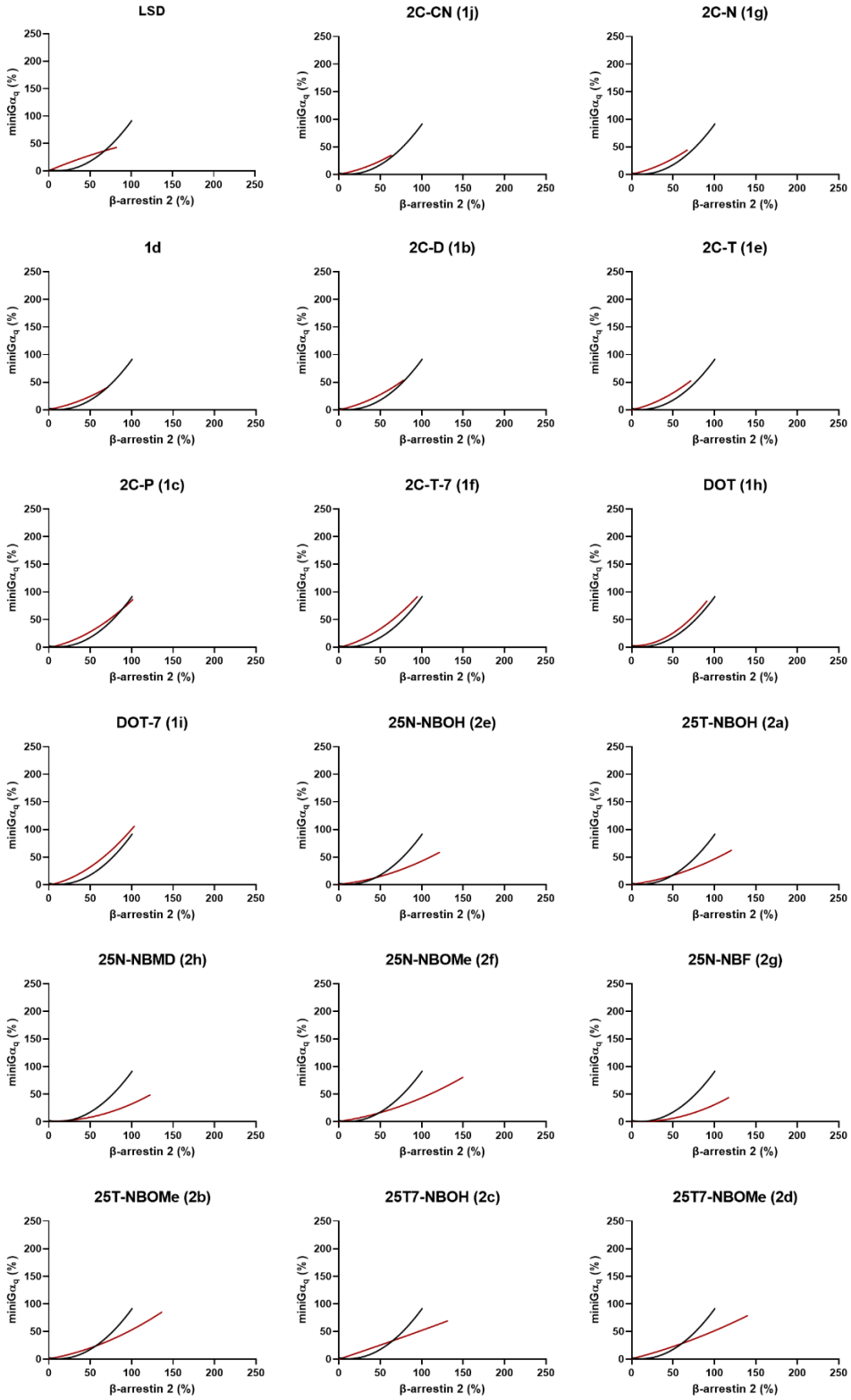


Figure S14: Qualitative bias plots, where each panel shows the centered second order polynomial fit of the activation values at equimolar concentrations of the substance in the respective assays in red, and that of the reference agonist (in this case serotonin) in black. The first 30 minutes of the time-luminescence profiles are used for the calculation of the AUC.

REFERENCES

(1) Pottie, E.; Cannaert, A.; Stove, C. P. In vitro structure-activity relationship determination of 30 psychedelic new psychoactive substances by means of beta-arrestin 2 recruitment to the serotonin 2A receptor. *Arch Toxicol* **2020**, *94* (10), 3449-3460. DOI: 10.1007/s00204-020-02836-w.
(2) Kim, K.; Che, T.; Panova, O.; DiBerto, J. F.; Lyu, J.; Krumm, B. E.; Wacker, D.; Robertson, M. J.; Seven, A. B.; Nichols, D. E.; et al. Structure of a Hallucinogen-Activated Gq-Coupled 5-HT2A Serotonin Receptor. *Cell* **2020**, *182* (6), 1574-1588 e1519. DOI: 10.1016/j.cell.2020.08.024.
(3) Eisenberg, D.; Schwarz, E.; Komaromy, M.; Wall, R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J Mol Biol* **1984**, *179* (1), 125-142. DOI: 10.1016/0022-2836(84)90309-7.